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A new *Arabidopsis thaliana* mutant deficient in the expression of *O*-methyltransferase impacts lignins and sinapoyl esters

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Abstract

A promoter-trap screen allowed us to identify an *Arabidopsis* line expressing GUS in the root vascular tissues. T-DNA border sequencing showed that the line was mutated in the caffeic acid *O*-methyltransferase 1 gene (*AtOMT1*) and therefore deficient in OMT1 activity. *Atomt1* is a knockout mutant and the expression profile of the *AtOMT1* gene has been determined as well as the consequences of the mutation on lignins, on soluble phenolics, on cell wall digestibility, and on the expression of the genes involved in monolignol biosynthesis. In this mutant and relative to the wild type, lignins lack syringyl (S) units and contain more 5-hydroxyguaiacyl units (5-OH-G), the precursors of S-units. The sinapoyl ester pool is modified with a two-fold reduction of sinapoyl-malate in the leaves and stems of mature plants as well as in seedlings. In addition, LC-MS analysis of the soluble phenolics extracted from the seedlings reveals the occurrence of unusual derivatives assigned to 5-OH-feruloyl malate and to 5-OH-feruloyl glucose. Therefore, AtOMT1 enzymatic activity appears to be involved not only in lignin formation but also in the biosynthesis of sinapate esters. In addition, a deregulation of other monolignol biosynthetic gene expression can be observed in the *Atomt1* mutant. A poplar cDNA encoding a caffeic acid OMT (*PtOMT1*) was successfully used to complement the *Atomt1* mutant and restored both the level of S units and of sinapate esters to the control level. However, the over-expression of *PtOMT1* in wild-type *Arabidopsis* did not increase the S-lignin content, suggesting that OMT is not a limiting enzyme for S-unit biosynthesis.

Introduction

Arabidopsis thaliana has been the subject of intense plant molecular research for over a decade. The identification of mutants has been an important approach that has led to the cloning of novel genes. This strategy has been very fruitful in some research topics such as hormone action and flower development. However, the use of *Arabidopsis* to study cell wall synthesis is somewhat limited (Reiter, 1998), mainly because of the difficulty in establishing efficient screening procedures. Most of the identified mutants are related to the formation of the primary cell wall (Fagard *et al.*, 2000). Screening tests for altered lignification have been recently considered (reviewed in Boudet, 2000) but, until now, very few reports relate the isolation

and characterization of the corresponding genes. The first *Arabidopsis* mutant reported to be altered in the lignification pathway was the *fah1* mutant, defective in ferulate 5-hydroxylase (F5H; Chapple *et al.*, 1992; Meyer *et al.*, 1996). Lignin composition in this mutant is highly modified with the complete absence of syringyl (S) units, the dimethoxylated component of lignin (Marita *et al.*, 1999) and the mutant is completely devoid of sinapoyl esters, Brassicaceae-specific compounds. Recently, Jones *et al.* (2001) demonstrated that the *irx4* (irregular xylem) mutant (Turner and Somerville, 1997) is deficient in cinnamoyl CoA reductase (CCR) and has a reduced lignin level.

Natural and chemically induced mutants with altered lignin level and/or structure have been isolated from other plant species and comprehensively analysed (reviewed in Barrière *et al.*, 1993; Baucher *et al.*, 1998). In a few cases, the mutated gene has been identified and cloned. For instance, the *brown-midrib* maize *bm1* and *bm3* mutations affect the cinnamyl alcohol dehydrogenase (CAD) gene (Halpin *et al.*, 1999) and the caffeic acid *O*-methyltransferase (COMT) gene (Vignols *et al.*, 1995), respectively. In addition, a spontaneous *Pinus taeda* CAD mutant has been characterized (Ralph *et al.*, 1996; MacKay *et al.*, 1997).

Arabidopsis has a typical dicot lignification pattern. The interfascicular fibres and the xylem bundles form a ring of lignified tissues in the mature stems (Dharmawardhana *et al.*, 1992). Lev-Yadun (1994) has shown that, when grown under special conditions, this annual dicot can produce secondary xylem in the flowering stem, as do woody plants.

T-DNA or transposon insertion lines seem very promising to identify novel secondary cell wall mutants. These mutants are easily identified from their phenotype and/or GUS expression pattern and the affected gene can be more rapidly cloned than when using chemically mutated lines (Parinov and Sundaresan, 2000). The Versailles collection of *Arabidopsis* T-DNA insertion mutants is therefore a good tool to screen for this mutant type. Lines of this collection contain, next to the T-DNA right border, the promoterless β-glucuronidase (*uidA* or *gus*) gene (Bouchez *et al.*, 1993). GUS activity in transgenic lines can be observed when T-DNA insertion induces a transcriptional or a translational fusion between the *gus* gene and an endogenous plant gene. In consequence, the *gus* reporter gene is under the control of the promoter of the tagged gene. A line was identified as expressing GUS in the root vascular tissues. The sequencing of the region linked to the insertion revealed that the *gus* gene was inserted as a translational fusion in the *O*-methyltransferase gene (*AtOMT1*). *O*-methylation, which is catalysed by *S*-adenosyl-L-methionine-dependent *O*-methyltransferases, involves a diverse range of plant phenolics including phenylpropanoids, coumarins and flavonoids (Ibrahim, 1997; Ibrahim *et al.*, 1998). The *AtOMT1* gene is highly related to those involved in the methylation of lignin precursors and therefore this line was investigated for lignin structure modifications. In this paper, we report the selection of the *Atomt1* mutant, the expression pattern of the tagged *OMT* gene and the consequences of this mutation on lignin structure. In addition, we have studied the impact of the mutation on the formation of sinapoyl esters, on the cell wall digestibility and on the expression of other genes of the lignin pathway. Finally, the effect of over-expressing a poplar OMT gene in the mutant and wild-type *Arabidopsis* lines was studied.

Materials and methods

Plant materials

The Versailles T-DNA insertion *A. thaliana* collection was used for the screening. In this collection, the plant transformation vector, pGKB5, designed for promoter trapping and gene tagging (Bouchez *et al.*, 1993) was used to transform wild-type *A. thaliana*, ecotype Wassilewskija (WS), by vacuum infiltration (Bechtold *et al.*, 1993). For the screening, seedlings originating from T_3 seeds were grown on Estelle and Somerville medium (1987) without selection and screened for GUS staining in roots as already described (Sarrobert *et al.*, 2000).

Histochemical localization of GUS activity and lignin staining

Beta-glucuronidase activity was assayed histochemically with X-Gluc according to Jefferson (1987) on 5-day old seedlings for screening and on different tissues of the mutant for expression studies. Free-hand cross-sections of floral stems were made. Wiesner and Mäule stainings were performed according to standard protocols. Photographs were taken under a binocular microscope.

Isolation and sequencing of T-DNA-flanking fragments

The DNA fragment corresponding to the right border sequence was amplified by the PCR walking method described by Devic *et al.* (1997). The BLAST search program (Altschul *et al.*, 1990) was used for sequence alignments. The multiple sequence alignment with hierarchical clustering (Coupet, 1988) was used to construct the phylogenetic tree.

Southern and northern analysis

Genomic DNA was isolated from seedlings or from greenhouse-grown plants as described by Doyle and Doyle (1990). Southern blots were performed according to Sambrook *et al.* (1989) with Gene Screen Plus membranes (Hybaid, Ashford, UK). Total RNA of different tissues were isolated as described by Verwoerd *et al.* (1989). The agarose gels and the RNA/DNA hybridization were performed according to Sambrook *et al.* (1989). Probes obtained after digestion of the plasmids containing the cDNA coding sequence or by PCR amplification were randomly primed in presence of α-^{32}P-dCTP. The *AtOMT1* cDNA was synthesized with the plasmid VBC-JG08 (accession number Z37679; Höfte *et al.*, 1993). The *AtF5H* probe corresponded to the *F5H* cDNA (Meyer *et al.*, 1996), the *AtCCoAOMT* probe to EST 120J2T7 (accession number T43508), the *CAD-D* probe to clone VBV-CE10 (accession number Z34154; Höfte *et al.*, 1993), and the *CAD-C* to clone *A*tciacde (Baucher *et al.*, 1994; accession number Z31715).

OMT activity

Crude proteins were extracted from seedlings, leaves and stems of *Arabidopsis* and quantified according to Bradford (1976). A 30 μg portion of total proteins from each organ was used. OMT enzyme assays were performed as previously described (Collendavelloo *et al.*, 1981; Van Doorsselaere *et al.*, 1995) with *S*-adenosyl-L-methionine (NEN, Boston, USA) and different substrates. Caffeic acid and quercetin were commercially obtained (Sigma, Steinheim, Germany). 5-OH-Ferulic acid, 5-OH-coniferaldehyde and 5-OH-coniferyl alcohol were synthesized as described below. Caffeic acid, 5-OH-ferulic acid and quercetin were used at a concentration of 3 mM, and 5-OH-coniferaldehyde and 5-OH-coniferyl alcohol at a concentration of 3 μM.

Synthesis of the 5-hydroxy substrates

5-Hydroxyvanillin, 3,4-dihydroxy-5-methoxybenzaldehyde, pyrrolidine, acetic anhydride, pyridine and triethyl phosphonoacetate were obtained from Aldrich Chemical Company (Milwaukee, WI). Flash chromatography used Biotage (Dyax, Cambridge, MA) Flash 40 cartridges (KP-SIL 60A, 32–63 μm silica) on a Flash 40 system, with an ISCO (Lincoln, NE) Foxy-200 fraction collector and an ISCO UA6 UV-VIS detector. Compound numbering used here is standard lignin numbering.

The 5-hydroxyguaiacyl substrates were prepared by various methods, some of them new. In particular, we favour cinnamyl alcohol synthesis via DIBAL reduction of the cinnamate ester at room temperature, which is itself formed from Wittig-Horner reaction of the corresponding benzaldehyde (see details below). Although a synthesis for the cinnamaldehyde has also been reported recently, we found that protection of the phenols and alcohol by t-butyldimethyl silylation and DDQ oxidation of the silyl ether to be a simpler route.

5-Hydroxyferulic acid

5-Hydroxyferulic acid was prepared from 5-hydroxyvanillin according to methods described (Lam *et al.*, 1996).

5-Hydroxyconiferyl alcohol

3,4-dihydroxy-5-methoxybenzaldehyde was acetylated with acetic anhydride/pyridine to produce 3,4-diacetoxy-5-methoxybenzaldehyde in essentially quantitative yield. Ethyl 4,5-diacetoxy-5-methoxyferulate was prepared by a Wittig-Horner reaction with triethyl phosphonoacetate (yield 90%) according to a previously described procedure (Ralph *et al.*, 1992). Ethyl 5-hydroxyferulate was obtained in a 92% yield by deacetylation with pyrrolidine. NMR: δ_H 3.86 (3H, s, methoxyl), 6.30 (1H, d, J = 15.8 Hz, β), 6.85 (1H, s, 6), 6.88 (1H, s, 2), 7.5 (1H, d, J = 15.8 Hz, β); δ_C 56.4 (OMe), 104.2 (2), 110.3 (6), 115.9 (β), 126.4 (1), 137.3 (4), 145.8 (α), 146.2 (5), 149.0 (3), 167.4 (δ). 5-Hydroxyconiferyl alcohol was synthesized (87% yield) from ethyl 5-hydroxyferulate by DIBAL-H reduction (Quideau *et al.*, 1992). NMR: δ_H 3.82 (3H, s, OMe), 4.18 (2H, dt, J = 5.6, 1.5 Hz, γ), 6.18 (1H, dt, J = 15.9, 5.6 Hz, β), 6.43 (1H, dt, J = 15.9, 1.5 Hz, α), 6.6 (2H, s, A2/A6); δC 56.4 (OMe), 63.4 (γ), 102.5 (2), 108.0 (6), 128.1 (β), 129.5 (1), 130.8 (α), 134.5 (4), 146.2 (A5), 149.0 (3).

5-Hydroxyconiferaldehyde

5-Hydroxyconiferyl alcohol was derivatized to its TBDMS derivative with TBDMS triflate and pyridine in methylene chloride. After work-up, the TBDMS-protected 5-hydroxyconiferyl alcohol was oxidized with DDQ (Paterson *et al.*, 1998) to the corresponding aldehyde in 79% yield after TLC purification. NMR: δ_H 3.89 (3H, s, OMe), 6.64 (1H, dd, $J = 15.9$, 7.6 Hz, β), 6.90 (1H, d, $J = 1.7$ Hz, 6), 7.07 (1H, d, $J = 1.7$ Hz, 2), 7.56 (1H, d, $J = 15.8$ Hz, a), 9.63 (1H, d, $J = 7.63$ Hz, γ); δC 55.8 (OMe), 105.7 (2), 116.0 (6), 127.99 (1), 128.01 (β), 140.2 (4), 148.5 (5), 153.3 (3), 153.7 (α), 193.7 (γ). Desilylation with sodium hydroxide in dioxane (Crouch *et al.*, 1999) produced 5-hydroxyconiferaldehyde in 62% yield after TLC purification. NMR: δ_H 3.89 (3H, s, OMe), 6.60 (1H, dd, $J = 15.8$, 7.63 Hz, β), 6.88 (1H, d, $J = 1.5$ Hz, 6), 6.95 (1H, d, $J = 1.5$ Hz, 2), 7.50 (1H, d, $J = 15.8$ Hz, α), 9.62 (1H, d, $J = 7.63$ Hz, γ); δC 56.6 (OMe), 104.7 (2), 111.1 (6), 126.5 (1), 127.2 (β), 138.3 (4), 146.5 (5), 149.2 (3), 154.4 (α), 193.8 (γ). The NMR data, fully authenticated by the usual complement of 1D and 2D methods, for the 5-hydroxyconiferaldehyde agreed with those recently published (Li *et al.*, 2000).

Lignin characterization

Lignin analyses were performed on dried stems. The lignin content of the extractive-free samples was estimated by the standard Klason procedure (Dence, 1992). Lignin structure was investigated with thioacidolysis and its most recent developments (Lapierre *et al.*, 1995). The lignin-derived monomers and dimers were identified by GC-MS of their TMS derivatives.

Extraction and HPLC analysis of the Arabidopsis *flavonoids and sinapoyl esters*

The extraction of soluble phenolics from the stems and leaves of mature plants and from seedlings was carried out in 80% v/v methanol according to a method adapted from Burbulis *et al.* (1996). 2′,3,4′,5′,7-pentahydroxyflavone (morin hydrate, Fluka, Buchs, Switzerland) was added to the extracts as an internal standard. The extracts were then analysed by LC-MS (electrospray ionization method, negative mode). The LC conditions were as follows: column, a HyPURITY-C18 column (ThermoHypersil, 4.6 mm × 150 mm, particle size 5 μm); flow rate 1 ml/min; linear elution gradient from 5% to 60% solvent B (CH_3CN + HCOOH, 0.1%) in solvent A (H_2O + HCOOH, 0.1%), within 30 min. The mass spectral data were collected with an ion trap mass spectrometer (Thermofinnigan LCQ Deca Instrument, Finnigan Mat, San Jose, CA) equipped with a heated capillary electrospray interface and in the negative mode. The sprayer needle voltage was 4 kV and the temperature of the heated capillary was set at 350 °C. The quantitative determination of sinapoyl malate was carried out relative to the morin hydrate internal standard by considering their relative surfaces of a particular ion on the LC-MS trace and with a response factor arbitrarily set at 1 as no authentic sinapoyl-malate was available. Such quantification was nevertheless valuable when comparing the samples.

Assessment of digestibility

The wild-type and mutant lines were sown in a glasshouse and, after emerging, plants were cropped in a growth chamber with the following conditions: 8 h day/16 h night, 20 °C day/16 °C night, relative humidity 80% and light intensity 160 μmol m^{-2} s^{-1}. Plants were harvested after 3 months in the growth chamber, after complete maturity of the floral stems. Different parameters of growth were recorded during growth or at harvest (flowering date, plant height, branch number and yield/plant). Floral stems were dried in an oven (65 °C) and dry samples were then ground with a hammer mill to pass through a 1 mm screen for later analyses. Neutral detergent fibre (NDF) was estimated according to Goering and van Soest (1970). *In vitro* dry matter digestibility (IVDMD) was estimated according to the enzymatic solubility of Aufrère and Michalet-Doreau (1983) and *in vitro* NDF digestibility (IVNDFD) was computed assuming that the non-NDF part of plant material was completely digestible, according to Struik (1983) and Dolstra and Medema (1990).

Transformation vector

The binary vector (35^2-SOMT) already used in Jouanin *et al.* (2000) was used. 35^2-SOMT contains the full-length coding sequence of the poplar (*Populus deltoides* × *P. trichocarpa*) COMT cDNA (Dumas *et al.*, 1992) under the control of the CaMV 35S promoter with a double enhancer sequence (Kay *et al.*, 1987). This chimeric gene was cloned in the pBIBHYG binary vector (Becker, 1990) containing a hygromycin resistance gene for the selection of transformed cells. This vector was introduced into the

Agrobacterium strain C58pMP90 (Koncz and Schell, 1986).

Generation and characterization of complemented and over-expressing lines

The *Agrobacterium* strain was used to transform *Arabidopsis* plants (ecotype WS) at the floral stages using the vacuum infiltration (Bechtold *et al.*, 1993) and the floral dip (Clough and Bent, 1998) methods. Seeds of these plants (T_0) were surface-sterilized and grown *in vitro* on Estelle and Somerville (1987) medium containing 1% sucrose, 0.7% agar and 50 μg/ml hygromycin. The transformed seedlings (T_1) were transferred into the greenhouse, allowed to self-cross and the seeds were harvested. T_2 plants containing one T-DNA insertion locus were selected *in vitro* as segregating on the selective medium with a 3:1 ratio. Eight of these plants were transferred to the greenhouse and T_3 homozygous plants were selected as presenting an entirely antibiotic-resistant progeny.

Results

Isolation of the Atomt1 *mutant*

A line of the Versailles collection of *Arabidopsis* T-DNA insertion mutants was selected through a screening for the activation of the GUS reporter gene (Sarrobert *et al.*, 2000) in the root vascular tissues at the seedling stage. DNA hybridization analysis with a fragment covering the right border showed that this line contains a single T-DNA insert (data not shown). The plant DNA adjacent to the *gus* coding sequence was sequenced by PCR walking (Devic *et al.*, 1997). The T-DNA integration has placed the ATG of the *gus* gene in frame with the first exon of an *OMT* gene and therefore allowed a functional β-glucuronidase gene fusion. A blast search in the NCBI database revealed a complete sequence identity with a caffeic acid *O*-methyltransferase cDNA (*AtOMT1*; accession number U70424) identified by Zhang *et al.* (1997). As foreseeable, the T-DNA segregated as a single copy and a homozygous line for the *AtOMT1* mutation was obtained. When cultivated in a greenhouse, this *Atomt1* line did not present phenotypic modifications: the growth and fertility were comparable to those of wild-type *Arabidopsis*.

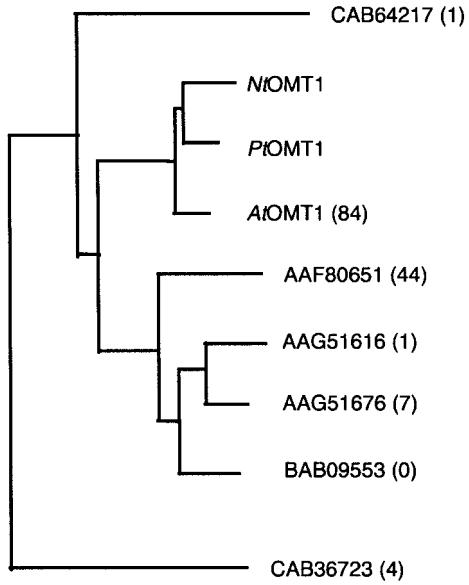

Figure 1. Putative phylogenetic tree of OMT genes from *Arabidopsis thaliana*, constructed from PAM matrix and the neighbor-joining algorithm. The tobacco (*NtOMT1*; Jaeck *et al.*, 1992) and the poplar (*PtOMT1*; Dumas *et al.* 1992) genes correspond to the sequences already used in antisense strategies (Atanassova *et al.*, 1995; Van Doorsselaere *et al.*, 1995). The number of identified ESTs in Genbank, for each Arabidopsis *OMT* gene is indicated in parenthesis.

Caffeate OMT-*like gene family in* Arabidopsis *and* AtOMT1 *gene expression in* Arabidopsis

Analysis of the *Arabidopsis* genome sequence (Arabidopsis Genome Initiative, 2000) revealed the presence of 6 other related sequences and a putative phylogenetic tree prediction from sequence multiple alignment by cluster and topological algorithms was generated (Figure 1). Among these *OMT* genes, the sequence of *AtOMT1* seems the most related to the caffeic acid/5-hydroxyferulate OMT cDNA (*COMT*) previously reported for other dicot plant species (tobacco, Jaeck *et al.*, 1992; poplar, Dumas *et al.*, 1992). A total of 84 ESTs corresponding to *AtOMT1* could be identified in different cDNA libraries corresponding to various organs such as roots, green siliques, above-ground organs, flower buds and developing seeds. Compared to the other *AtOMT1*-related genes, *AtOMT1* corresponds to the most frequently reported EST (Figure 1). Another putative *OMT* cDNA, corresponding to protein AAF80651, is also frequently observed in EST databases in the same organs (44 ESTs). To investigate the expression of *AtOMT1* in wild-type *Arabidopsis*, we carried out RNA/DNA hybridizations and examined the GUS expression profile.

Table 1. Percentage of OMT activity in stem extracts using different substrates in the wild type, the mutant (*Atomt1*) and the over-expressing line, SOMT-22. Activity against quercetin and 5-OH ferulic acid are expressed as a percentage relative to caffeic acid activity in the wild type. Activity against 5-OH-coniferaldehyde and 5-OH-coniferyl alcohol are expressed as a percentage of activity in the wild type for the two substrates. Results are the means of two experiments repeated three times.

Line	Caffeic acid (3 mM)	5-OH-ferulic acid (3 mM)	Quercetin (3 mM)	5-OH-coniferaldehyde (3 μM)	5-OH-coniferyl alcohol (3 μM)
Wild type	100%	42.8 ± 0.4%	53.4 ± 1.3%	100%	100%
Atomt1	6.6 ± 0.4%	6.9 ± 1.1%	0.6 ± 0.1%	14.9 ± 0.8%	17.0 ± 0.5%
SOMT-22	572.0 ± 9.8%	218.8 ± 1.2%	79.5 ± 2%	285.3 ± 4.6%	222.5 ± 0.9%

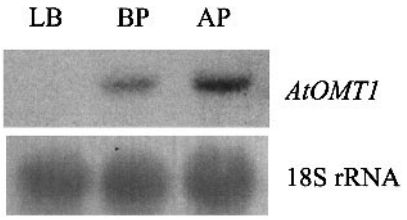

Figure 2. RNA gel blot analysis of different tissues from wild-type *Arabidopsis*; LB, leaf blade; LB, basal part of the stem; AP, apical part of the stem. The blot was probed with the full-length *AtOMT1* sequence at high stringency. Hybridization with the 18S rDNA was used to estimate RNA loading.

Northern experiments were performed with total RNA from leaf blade and from apical and basal parts of the floral stems of wild-type plants with a PCR probe corresponding to the *AtOMT1* coding sequence. The signal was barely detectable in the leaf blade. It was found higher in the apical than in the basal part of the stems (Figure 2).

Thanks to the GUS fusion, *AtOMT1* expression could be determined by histochemical observations of various tissues. At the young seedling stage (3 days after germination), GUS activity was found to be constitutively expressed in the whole plant. During the next days, the constitutive expression gradually faded and in older seedlings (12 days), GUS expression was mainly restricted to vascular tissues of root, cotyledons and leaves. A vascular pattern associated with a basal GUS activity in leaf blade was detected in young leaves whereas the GUS activity was restricted to the vascular tissues in mature leaves. Xylem, mature phloem and differentiating fibres were the main sites of GUS activity in the stems. In flowers, the GUS expression was observed in sepal veins but not in the other parts of the flowers including petals or pollen. In siliques, GUS expression was restricted to the lignified extremities (Figure 3).

OMT activity in the Atomt1 *mutant*

Different compounds assumed or known to be OMT substrates were used to determine if the *Atomt1* mutant was defective in the methylation of lignin precursors. The compounds conventionally used for such analysis, caffeic acid and 5-hydroxyferulic acid, were tested (Atanassova *et al.* 1995; Van Doorsselaere *et al.*, 1995; Jouanin *et al.*, 2000). A very low residual activity for both substrates was observed in the mutant line when compared to the wild type (Table 1). OMT activity was high in seedlings and floral stems and low in leaves of the wild type (data not shown). Several recent studies have proposed 5- hydroxconiferaldehyde and 5-hydroxyconiferyl alcohol as the preferred COMT substrates (Humphreys *et al.*, 1999; Osakabe *et al.*, 1999; Maury *et al.*, 1999; Li *et al.*, 2000; Parvathi *et al.*, 2001). In our hands, these substrates could be successfully employed with wild-type plant extracts at a lower concentration than the corresponding acids in agreement with literature data (Li *et al.*, 2000). The mutant had a reduced ability to use these substrates as well (Table 1). A recent publication (Muzac *et al.*, 2000) suggested that this OMT could use the flavanol quercetin as the preferred substrate. While wild-type seedlings and stems were capable of methylating quercetin, the mutant samples lacked this ability (Table 1).

Lignification of floral stems

Stem sections of the wild type and the mutant were compared after specific lignin staining. No coloration difference was detected after Wiesner staining. In contrast, Mäule coloration, which is more specific of S-lignin units, revealed striking differences (Figure 4A and 4B). Whereas wild-type samples exhibited the positive purple coloration diagnostic of S units, in the

Table 2. Lignin content (Klason lignin, KL, as a percentage of the dry weight of the extract-free stems) and yields (μmol per g KL) of the H, G, S and 5-OH-G thioacidolysis monomers and sinapoyl-malate content (ng per mg fresh weight) recovered from the stem of the wild type, the mutant (*Atomt1*), a fully complemented (CpOMT-14) and over-expressing (SOMT-22) *Arabidopsis* lines. Each transformed line has its own control grown under exactly the same conditions. The monomers, analysed by GC-MS of their silylated derivatives, are estimated from the areas of their specific base peak ions on reconstructed ion chromatograms. The relative standard errors between duplicate thioacidolysis experiments, which are not reported for the sake of clarity, do not exceed 3%. Sinapoyl-malate content was estimated by measuring the areas of the peaks relative to that of the internal standard and without correction for LC-MS response factors. The reported values, which are the mean of three measures on different plants of each line, are therefore discussed in a comparative way and not on the basis of absolute content. nd, not determined.

	Wild type	*Atomt1*	CpOMT14	SOMT-22
Klason lignin (KL)	14.23	15.51	–	–
	13.66	–	13.41	–
	15.20	–	–	14.31
H (μmol per gram KL)	13	11	–	–
	17	–	13	–
	18	–	–	15
G (μmol per gram KL)	986	678	–	–
	928	–	831	–
	1012	–	–	982
S (μmol per gram KL)	330	**11**	–	–
	341	–	241	–
	371	–	–	373
5-OH-G (μmol per gram KL)	8	**20**	–	–
	6	–	6	–
	5	–	–	3
Total monomer yields (μmol per gram KL)	1337	**720**	–	–
	1292	–	1091	–
	1406	–	–	1373
Sinapoyl-malate in leaves (ng/mg)	157 $\pm$ 23	70 $\pm$ 13	178 $\pm$ 4	nd
Sinapoyl-malate in stems (ng/mg)	73 $\pm$ 12	34 $\pm$ 6	115 $\pm$ 23	nd

interfascicular fibres, the mutant line displayed a negative light brown coloration. This observation suggests that S-lignin units are scarce or absent in the mutant.

The lignin content of extractive-free stems was determined by the Klason method with different cultures of wild-type and mutant plants. A slight increase in Klason lignin was observed in *Atomt1* (Table 2). Lignin-derived monomers released by thioacidolysis were analysed by gas chromatography-mass spectrometry (GC-MS) for the wild-type line and for the mutant line at the hemizygous and homozygous stages. No difference between the wild-type and hemizygous *Atomt1* lines could be detected (data not shown). In contrast, the homozygous *Atomt1* line displayed a dramatically altered profile, with an increase in G and in 5-hydroxyguaiacyl (5-OH-G) monomers and a S monomer level close to zero (Table 2). In addition and relative to the wild type, this line released a lower amount of thioacidolysis monomers when yields are expressed on the basis of the lignin content (Table 2). Lignin-derived dimers obtained by thioacidolysis followed by Raney nickel desulfurization were also examined by GC-MS. In agreement with the analysis of thioacidolysis monomers, SG or

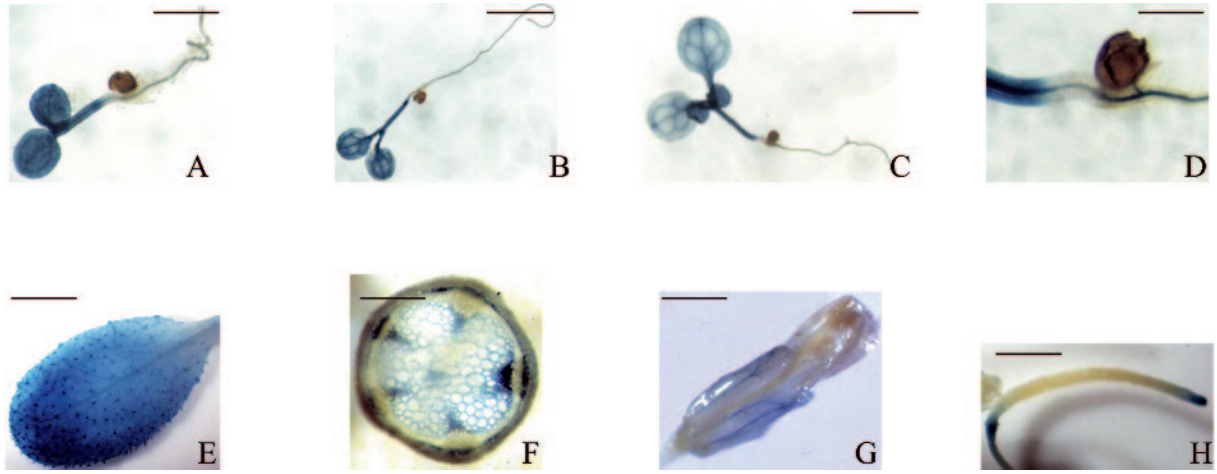

Figure 3. Analysis of *AtOMT1* promoter-driven GUS expression in different tissues and developmental stages of the *Atomt1* mutant. A. Three days after germination (bar 5 mm). B. Seven days after germination (bar 1 cm). C. Twelve days after germination (bar 5 mm). D. Detail of B (bar 1 mm). E. Young leaf (bar 5 mm). F. Stem section (bar 1 mm). G. Floral bud (bar 0.5 mm). H. Silique (bar 2 mm).

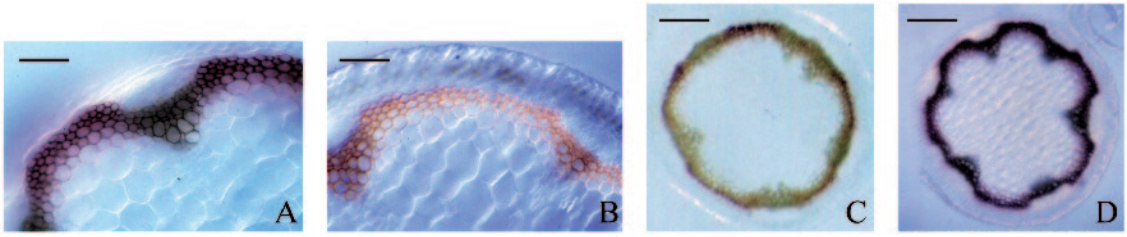

Figure 4. Histochemical staining of stem sections with Mäule reagent. A. Wild type. B. *Atomt1* (bar 0.1 mm). C, D. A partly (C, CpOMT-7) and a fully (D, CpOMT-14) complemented line (bar 0.5 mm).

SS dimers were found absent in the case of the homozygous *Atomt1* line (data not shown). As evidenced in COMT-deficient poplar (Jouanin *et al.*, 2000; Ralph *et al.*, 2001a, b), a novel dimer corresponding to a benzodioxane structure involving a G-unit and a 5-OH-G unit was observed (data not shown).

Soluble flavonoids and sinapoyl esters from wild-type and Atomt1 *lines*

Muzac *et al.* (2000) reported that the OMT corresponding to the currently affected gene could methylate quercetin to produce isorhamnetin. Since *in vitro* activity assays revealed that the mutant line could not methylate quercetin in contrast to the wild-type line, soluble phenolics were extracted from mutant and wild-type samples according to a method adapted from Burbulis *et al.* (1996) and analysed by liquid chromatography-mass spectrometry (LC-MS) (electrospray ionization, negative mode). Irrespective of the line and in agreement with literature data (Graham, 1998), the main flavonoids recovered from mature floral stems and from leaves collected at the flowering stage were kaempferol glycosides, with a predominance of kaempferol dirhamnoside (data not shown). Quercetin glycosides were recovered as minor components while they were observed to occur in higher relative amount in seedlings, in agreement with literature data (Pelletier *et al.*, 1999). Whatever the sample, only trace amounts of isorhamnetin derivatives could be seen. No evidence was therefore found for a significant difference in the flavonoid machinery between the wild-type and mutant lines. It was previously reported that the *fah1* mutant, deficient in ferulate 5-hydroxylase (F5H) activity, completely lacked S units in lignins and also soluble sinapoyl esters in leaves (Chapple *et al.*, 1992, Meyer *et al.*, 1996). To investigate the possible involvement of *At*OMT1 in the pathway leading to sinapoyl esters, we examined the sinapoyl derivatives extracted from fresh wild-type and mutant samples (stems and leaves collected at the flowering stage as well as seedlings) by LC-MS (Table 2 and Figure 5). Sinapoyl-malate (SM) was recovered from fresh and green tissues as a mixture

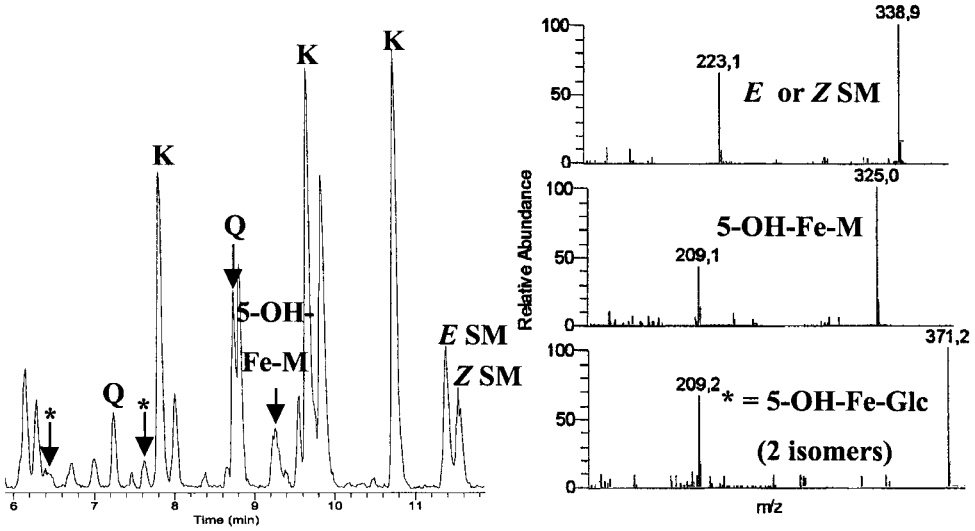

Figure 5. LC-MS traces showing the separation of soluble phenolic compounds recovered from *Atomt1* seedlings. Peaks corresponding to kaempferol glycosides (K) and to quercetin glycosides (Q), as revealed by their characteristic mass spectra, do not allow discrimination between the *Atomt1* mutant and the wild-type samples. In contrast, peaks corresponding to the (*E*) and (*Z*) isomers of sinapoyl-malate (*E* SM and *Z* SM) are reduced in the mutant line while novel peaks tentatively assigned to 5-OH-feruloyl malate (5-OH-Fe-M) and to 5-OH-feruloyl glucose (5-OH-Fe-Glc, 2 isomers), on the basis of their mass spectra (electrospray ionization, negative mode) can be observed.

of (*E*) and (*Z*) isomers (Figure 5) whereas released in only trace amounts from mature dried stems. Their identity was established from their characteristic mass spectra (electrospray, negative mode) displaying a (M-H) ion at *m/z* 339 and the sinapate ion at *m/z* 223. Photoisomerization experiments induced a relative increase of the minor (*Z*) isomer. Sinapic acid was recovered after alkaline hydrolysis. When compared to the wild type, a two-fold reduction in the content of SM released from the leaves and stems of *Atomt1* plants collected at the flowering stage was observed (Table 2). The SM level was not reduced to zero in the *Atomt1* mutant, in contrast to the *fah1* mutant. In addition on the LC-MS trace of the soluble phenolics recovered from *Atomt1* seedlings, novel compounds that could be assigned to 5-hydroxyferuloyl derivatives on the basis of their mass spectra were observed. 5-Hydroxyferuloyl-malate (an (M-H) ion at *m/z* 325 and a 5-OH-ferulate ion at *m/z* 209) was observed as a peak with about half the area of the sinapoyl-malate peak, and two derivatives tentatively assigned to 5-hydroxyferuloyl-glucose isomers (an (M-H) ion at *m/z* 371 and a 5-OH-ferulate ion at *m/z* 209) were observed as minor peaks (Figure 5). These 5-OH-feruloyl derivatives were not detectable in wild-type samples, regardless of the developmental stage, and could be only observed as trace components in the fresh stems and leaves from mature *Atomt1* plants.

Cell wall digestibility

To evaluate the *in vitro* digestibility of cell walls, wild-type and *Atomt1* plants were grown at a larger scale in a growth chamber. The various traits related to plant growth and development did not allow the discrimination of the wild-type and mutant lines (Table 3). The *in vitro* digestibility of the dry matter (IVDMD) was measured by enzymatic treatment of the mature floral stems. When calculated on the basis of the neutral detergent fibre (NDF) content, *in vitro* NDF digestibility (IVNDFD) of the mutant stem was found to be slightly higher compared to the wild-type stem (Table 3). This difference, which was not significant, is however in agreement with data observed on *bm3* maize mutant and suggested that the *Atomt1* mutant cell walls were more susceptible to enzymatic hydrolysis than the wild-type ones.

Expression of other lignin monomer biosynthesis genes in Atomt1

Previous studies (Martz *et al.*, 1998; Pinçon *et al.*, 2001a, b) have shown that the down-regulation of one gene of the lignin biosynthesis pathway may affect the expression of other genes of this pathway. We therefore examined the transcript accumulation of ferulate 5-hydroxylase (*F5H*), caffeoyl-CoA *O*-methyltransferase (*CCoAOMT*) and cinnamyl alco-

Table 3. Growth characteristics and digestibility of the wild-type and the *Atomt1* mutant. DM, dry matter; IVDMD, *in vitro* dry matter digestibility; NDF, neutral detergent fibre; IVNDFD, *in vitro* NDF digestibility. Assays were performed on 30 plants from each line.

Line	Wild type	Atomt1
Biomass yield/plant (g DM)	0.28 + 0.03	0.30 + 0.03
Floral stem height (cm)	59.9 + 2.8	60.1 + 2.8
IVDMD %	54.4 + 1.8	56.1 + 1.8
NDF %	55.8 + 1.6	57.7 + 1.6
IVNDFD	18.2 + 1.9	21.2 + 1.9

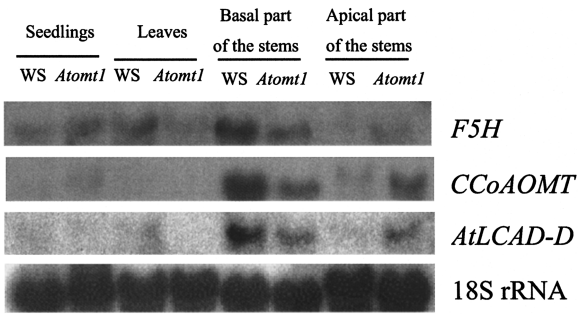

Figure 6. RNA gel blot analysis of different tissues from wild-type (WS) and *Atomt1* (*Atomt1*) plants probed with cDNA probes corresponding to F5H, CCoAOMT and AtLCAD-D. Hybridization with the 18S rDNA allowed the estimation of the RNA loading.

hol dehydrogenases (*CAD*) in wild-type and mutant plants. Total RNA extracted from different tissues (seedling, leaf, basal and apical parts of the stems) were blotted and hybridized to probes corresponding to the coding sequences of *F5H*, *CCoAOMT*, *AtCAD-C* and *AtCAD-D* (Figure 6). All four genes were found to be poorly expressed in seedlings and leaves, and highly expressed in the basal part of the stems in wild-type plants. In contrast, the expression levels of *F5H*, *CCoAOMT* and *AtCAD-D* genes were found to be reduced in the basal part of the *Atomt1* stems but up-regulated in the apical part of the stems. No difference in the expression profiles of the wild type and the mutant were observed when the *AtCAD-C* probe was used (result not shown).

Complementation of the Atomt1 *mutant with the* PtOMT1 *gene*

A construct containing the poplar *PtOMT1* cDNA under the control of a constitutive promoter (Jouanin *et al.*, 2000) was introduced into the *Atomt1* mutant to explore the restoration of S-unit deposition and sinapoyl-malate content. Transgenic lines segregating in a 3:1 ratio (one T-DNA insertion locus) were selected and homozygous plants were obtained (named CpOMT lines). Lines complementing partially (CpOMT7) the mutant phenotype or restoring the wild-type phenotype (CpOMT14) were identified using Mäule staining (Figure 4C and 4D) and by measuring OMT activity against caffeic acid (data not shown). Lignification in dried stems of CpOMT14 complementing the mutation was examined (Table 2). The S-unit frequency and the lignin content of the CpOMT-14 line was restored to the wild-type level, establishing the efficiency of the used poplar OMT construct. In addition, the wild-type level of sinapoyl-malate could be restored in the leaves and the stems of the CpOMT14 line (Table 2).

Over-expression of PtOMT1 *in wild-type* Arabidopsis

In order to determine if *At*OMT1 activity is a limiting factor in the accumulation of S units, the same construct as described above (Jouanin *et al.*, 2000) was introduced into wild-type *Arabidopsis*. Homozygous plants (SOMT lines) containing one insertion locus were selected and OMT activity was determined using caffeic acid as substrate. Three lines (SOMT-20, -22 and -24) possessing more than 200% OMT activity in stem extracts when compared to wild-type plants were selected for lignin analysis. The ability to use the preferred substrates was determined on stem extracts of SOMT-22 line, which had a high activity with caffeic acid (212% of wild-type activity). Increased OMT activities in this over-expressing line were observed not only with caffeic acid, but also with 5-hydroxyferulic acid, 5-hydroxyconiferaldehyde, 5-hydroxyconiferyl alcohol and quercetin (Table 1). This result demonstrates that the poplar OMT can use the same substrates as the *Arabidopsis* one. Wild-type and SOMT-22 lines displayed similar lignin contents as the corresponding control (Table 2). In agreement with Mäule staining (data not shown), thioacidolysis revealed that S-units occurred with the same frequency and in the same tissues in these lines as in the wild-type line (Table 2).

Discussion

Isolation of the Atomt1 *mutant and the* OMT *multigene family in* Arabidopsis

The *Atomt1* mutant line was identified in the Versailles T-DNA insertion collection based on the expression of the β-glucuronidase reporter gene in root vascular tissues. The tagged gene encodes a potential caffeic acid *O*-methyltransferase (*AtOMT1*). Due to its high homology with an OMT involved in the lignification process in tobacco, poplar and alfalfa (Gowri *et al.*, 1991; Dumas *et al.*, 1992; Atanassova *et al.*, 1995), we suspected that the absence of this enzyme activity could induce large changes in lignin structure as already observed in COMT-deficient plants (maize *bm3* mutant: Lapierre *et al.*, 1988; Vignols *et al.*, 1995, tobacco: Atanassova *et al.*, 1995, poplar: Van Doorsselaere *et al.*, 1995; Tsai *et al.*, 1998, Jouanin *et al.*, 2000, Ralph *et al.*, 2001a, b; alfalfa: Guo *et al.*, 2001a). Similar to the *fah1* mutant mutated in the *F5H* gene (Chapple *et al.*, 1992), the growth traits (size, flowering time, etc.) of the null-recessive *Atomt1* mutant in greenhouse conditions were comparable to those of the wild-type plant. In both cases, the mutations have no substantial impact on lignin content, but major consequences on lignin structure. While COMT deficiency is associated with the brownish coloration of the veins in *bm3* maize mutants (Barrière and Argillier, 1993) and of the xylem of transgenic poplars (Van Doorsselaere *et al.*, 1995; Tsai *et al.*, 1998; Jouanin et al., 2000), no coloration could be observed in *Atomt1* (this work) or in OMT down-regulated tobaccos (Atanassova *et al.* 1995), even when cross-sections of the stems were observed under high magnification.

The tagged gene is present as a single copy in the *Arabidopsis* genome. However, computer analysis of the complete *Arabidopsis* sequence demonstrated that this *OMT* gene belongs to a small multigene family. The homology between *AtOMT1* and the other genes of the *OMT* family is low. Therefore, these sequence divergences do not give any indication of the function of the related genes. *O*-Methyltransferases are involved in many different pathways and relatively minor sequence modifications can result in a modification in substrate affinity (Gauthier *et al.*, 1998; Wang and Pichersky, 1999).

Expression pattern of AtOMT1

In a previous report, expression of *AtOMT1* at the RNA level was determined in some tissues and leaf developmental stages (Zhang *et al.*, 1997). We have completed this study by analysing the expression in the lower (already lignified) and the upper (undergoing lignification) parts of stems and in the leaf blade (non-lignified tissue). The expression level was high in tissues under active lignification. These results are in accordance with those obtained for alfalfa (Gowri *et al.*, 1991), poplar (Bugos *et al.*, 1991), maize (Collazo *et al.*, 1992) and tobacco (Jaeck *et al.*, 1992) plants.

In addition, by taking advantage of the translational GUS fusion present in the mutant, the GUS expression pattern was determined by histochemistry. At the young seedling stage, GUS expression was constitutive, but decreased during development and at an older stage it was mainly localized to the vascular tissues of cotyledons, leaves and root. Xylem-specific expression was confirmed in floral stems since GUS was localized to the xylem and the interfascicular fibres. GUS expression found at the extremities of siliques correlated with lignification. Similar to maize COMT (Capellades *et al.*, 1996), *At*OMT1 expression seems therefore closely associated with lignification in *Arabidopsis*. However, the exclusive involvement of OMT in S-unit synthesis does not fit with the expression profile observed in young seedlings and suggests that this OMT could be involved in other pathways. The early *AtOMT1* expression is supported by the identification of the corresponding cDNA among ESTs randomly sequenced from a cDNA library made with 5-day old seedlings (Höfte *et al.*, 1993). The low constitutive expression level observed herein could be related to the high EST number (84) that we could identify in public cDNA libraries made from various tissues. Another *OMT* cDNA was frequently observed in these libraries, which is probably involved in the methylation of substrates other than lignin-related ones. Further investigations would be necessary to elucidate the function of this gene and of the 5 other *OMT* genes, which are present in the *Arabidopsis* genome but are expressed at low level or under specific conditions or tissues.

Substrate affinity of At*OMT1*

Experiments were undertaken to determine OMT activity in different tissues first with caffeic acid as substrate. In wild-type *Arabidopsis*, in accordance with

mRNA expression, caffeic acid OMT activity was high at the seedling stage and in floral stems but low in mature leaves. The mutant possesses a very low ability to methylate caffeic acid and 5-hydroxyferulic acid as already observed in antisense COMT plants (Atanassova *et al.*, 1995; Van Doorsselaere *et al.*, 1995). These substrates are not considered to be the *in vivo* ones for the synthesis of lignin precursors. Therefore, 5-hydroxyconiferaldehyde and 5-hydroxyconiferyl alcohol (Humphreys *et al.*, 1999; Osakabe *et al.*, 1999; Li *et al.*, 2000; Parvathi *et al.*, 2001) were tested. The high activity observed with these substrates confirmed that *AtOMT1* also used them. The mutant displayed a reduced capability to methylate these substrates as well, which further establishes the role of *At*OMT1 in the methylation of the S-monolignol precursor. Muzac *et al.* (2000) have recently reported that *At*OMT1, when expressed in *Escherichia coli*, essentially methylates the flavanol quercetin while caffeic and 5-hydroxyferulic acids were not used to any significant extent. We compared the ability of wild-type and mutant extracts to methylate quercetin, and indeed, in agreement with Muzac *et al.* (2000), we observed that the mutant has lost this capability. *At*OMT1 could be therefore involved not only in lignin, but also in flavonoid metabolism.

At*OMT1 deficiency and lignification*

The frequency of S monomers recovered following thioacidolysis from the mutant lignins was close to zero. In contrast, 5-OH-G monomers were recovered in this line, while released in trace amounts from the control. This trait has been repeatedly reported to be specific of the lignins in plants with depressed COMT, such as maize bm3 mutant (Lapierre *et al.*, 1988), transgenic tobaccos (Atanossava *et al.*, 1995), transgenic poplars (Van Doorsselaere *et al.*, 1995; Tsai *et al.*, 1998; Jouanin *et al.*, 2000) and transgenic alfalfa (Guo *et al.*, 2001a). Recent studies revealed that these diagnostic 5-OH-G units are mainly involved in the formation of novel benzodioxane structures (Jouanin *et al.*, 2000; Ralph *et al.*, 2001a). This novel bonding pattern specific for 5-OH-G units was confirmed in the case of the *Atomt1* mutant by the evidence of the diagnostic benzodioxane dimer released by thioacidolysis.

In angiosperms, caffeic acid *O*-methyltransferases (COMT) were previously considered to be bispecific involved in the methylation of both caffeic acid and 5-hydroxyferulic acid (Bugos *et al.*, 1992; Van Doorsselaere *et al.*, 1993). However, two *O*-methyltransferases (COMT and CCoAOMT) are now considered to be involved in constitutive lignification. CCoAOMT is involved in the biosynthesis of guaiacyl (G) lignin precursors (Ye *et al.*, 1995; Zhong *et al.*, 1998; Maury *et al.*, 1999; Guo *et al.*, 2001a) which is why the reduced AtOMT1 activity did not affect G unit synthesis in *Atomt1*.

The small increase in the Klason lignin level observed for the mutant line could be related to the enrichment in G-units, as G lignins are less acid-soluble than G-S lignins (Musha and Goring, 1974). In contrast, Marita *et al.* (1999) observed a large decrease in lignin content in *Arabidopsis* lines over-expressing F5H and a shift toward S lignin-type. No change in lignin content has been observed when caffeic acid OMT activity was reduced in tobacco (Atanassova *et al.*, 1995) and poplar (Van Doorsselaere *et al.*, 1995; Tsai *et al.*, 1998) plants. Lignin reduction has been found in a few cases such as in COMT-silenced poplars (Jouanin *et al.*, 2000), in *bm3* maize (Lapierre *et al.*, 1988; Barrière and Argillier, 1993) and antisense- COMT alfalfa (Guo *et al.*, 2001a).

At*OMT1 deficiency and soluble phenolics*

Even if the mutant protein extract had lost the ability to methylate quercetin *in vitro*, no clear-cut difference could be detected between the flavonoid profiles of the wild-type and the *Atomt1* stems and leaves, when grown under standard conditions. In contrast, the sinapoyl- malate level in the stems and in the leaves was strongly reduced in the *Atomt1* mutant, suggesting the involvement of *At*OMT1 in sinapoyl ester biosynthesis as already demonstrated for F5H (Chapple *et al.*, 1992; Ruegger *et al.*, 1996). The fact that sinapoyl-malate content is not reduced to zero (as it is in the F5H mutant) in this *Atomt*1 null mutant suggests the involvement of other *Arabidopsis OMT* genes in the pathway leading to sinapoyl esters. It is now accepted that lignification-specific OMT acts at the alcohol or aldehyde level. Our data provide new evidence to broaden this assessment as *At*OMT1 has a role in sinapate ester metabolism and therefore also acts at the acid level in *Arabidopsis thaliana*. In addition, the accumulation of 5-hydroxyferuloyl-malate and 5-hydroxyferuloyl-glucose in seedlings suggests that the enzymes responsible for the conversion of sinapate to sinapoyl-malate (UDP-glucose:sinapoyltransferase and sinapoyl-glucose:malate sinapoyltransferase) are also active with 5-hydroxyferulate which accumulates

as a consequence of the absence of *At*OMT1 activity in seedlings.

OMT could have different roles or substrate affinities according to tissues or to plant maturity as already suggested for wheat seedlings (Lam *et al.*, 1996) and for alfalfa (Inoue *et al.*, 1998). In addition, several reports demonstrate the induction of OMT by wounding and by elicitors (Gowri *et al.*, 1991; Jaeck *et al.*, 1992; Capellades *et al.*, 1996; Sibout *et al.*, unpublished results). The role of OMT in pathways other than the monolignol one needs to be further addressed. A re-examination of the profile of flavonoid- and sinapate-derived products of various plants deregulated for OMT activity under different conditions would be helpful.

Cell wall digestibility of the Atomt1 *mutant*

The improved cell wall digestibility of the bm3 mutant maize is well established (Barrière and Argillier, 1993). COMT antisense tobacco plants (Bernard-Vaihé *et al.*, 1996; Sewalt *et al.*, 1997), and recently both COMT and CCoAOMT antisense alfalfa transgenics (Guo *et al.*, 2001b), have also been reported to be more digestible. In this context, it was of interest to evaluate the digestibility of this novel *Arabidopsis* mutant. The cell walls of this *Arabidopsis* mutant were found slightly more susceptible to enzymatic hydrolysis and displayed a higher digestibility, relative to the wild-type line. In the case of maize, the improved digestibility was mainly assigned to the lower lignin level, rather than to the alteration in lignin structure (Sewalt *et al.*, 1997). In our mutant, as well as in the COMT antisense tobacco plants analysed by Bernard-Vailhé *et al.* (1996) and the alfalfa plants analysed by Guo *et al.* (2001b), the improvement of digestibility may be less unambiguously assigned to the lignin structure alteration. This result suggests that similar causal relationships between lignin structure and cell wall digestibility are observed in *Arabidopsis* and in tobacco and alfalfa plants. This similarity therefore validates the interest in *Arabidopsis* as a model plant to study not only lignification, but also cell wall digestibility, which is an important issue for ruminant animal performance.

Consequences of AtOMT1 *absence on expression of other genes*

Previous studies (Martz *et al.*, 1998; Pinçon *et al.*, 2001a; 2001b; Goujon, personal communication) have shown that down-regulating one target gene of the lignin biosynthetic pathway can affect the expression of other genes of this pathway. We have therefore examined the transcription level of *F5H*, *CCoAOMT* and *CAD-C* and *-D* genes in the Atomt1 line. The expression pattern of *F5H*, *CCoAOMT* and *CAD-D* genes changes, with a lower expression in the basal part of the stem and a higher one in the apical and youngest part. This observation reveals that the mutant has a modified lignification time schedule. Transmission electron microscopy observations of COMT down-regulated tobaccos showed that vessels of the transgenic plant seem immature when compared to the wild type (Ruel *et al.*, 2001). The expression pattern of the genes involved in the monolignol biosynthetic pathway of the mutant could suggest the same phenomenon: a delay in the expression of the other monolignol biosynthetic genes. It must be noticed that the reduction of CCoAOMT expression could be involved in the large decrease of total OMT activity measured with different substrates in the *Atomt1* mutant.

Complementation of the mutant and over-expression of OMT in the wild type

The functional complementation of the mutant with the poplar *OMT* cDNA, already used in a previous study on poplar (Jouanin *et al.*, 2000), demonstrates that the two genes are orthologous. The S content in some complemented lines is restored to the wild-type level, as well as the sinapoyl-malate content. The fact that a poplar enzyme could restore a biosynthetic pathway present only in Brassicaceae shows that the specificity of the sinapoyl ester pathway is not due to this OMT activity. Large over-expression of this poplar construct in wild-type *Arabidopsis* does not substantially increase the S frequency in lignins or the sinapoyl-malate content in the stem or in the leaves. In addition, the S-lignin deposition pattern, observed by Mäule staining, is similar in wild-type, complemented and over-expressing lines, without any S units in the xylem. This observation contrasts with those made when F5H is over-expressed in the *fah1* mutants deficient in this activity (Meyer *et al.*, 1998), in which case S units are synthesized not only in the fibres (complementation of the mutation), but also in the xylem where they are normally absent. These results demonstrate that OMT, in contrast to F5H (Meyer *et al.*, 1998; Franke *et al.*, 2000; Sibout *et al.*, 2002), is not a limiting enzyme for the biosynthesis of S units or of sinapoyl-malate. However, when F5H is highly expressed, the methylation step encoded by OMT turns

to be limiting, as 5-OH-G accumulates and is incorporated in benzodioxane structures (Ralph *et al.*, 2001b). The slight reduction of 5-OH-G monomers released by *PtOMT1* over-expressing lines has to be confirmed (Table 2), but nevertheless suggests that the co-expression of F5H and OMT could be a good strategy to increase S units in plant lignins. In this study, a poplar cDNA with 79% identity to *Arabidopsis OMT1* cDNA was chosen to avoid silencing effects which are frequent when over-expression is performed in homologous systems (Atanossova *et al.*, 1995; Tsai *et al.*, 1998; Jouanin *et al.*, 2000). This strategy proved to be successful in the selected *Arabidopsis* lines over-expressing the poplar OMT.

Conclusion

This work establishes the participation of *At*OMT1 in the biosynthesis of the S units of *Arabidopsis* lignins and of soluble sinapoyl esters. However, while the null *Atomt1* mutant lacks S units, the sinapoyl esters are not reduced to zero. The partial involvement of *At*OMT1 in sinapoyl ester metabolism very likely explains the expression pattern of *AtOMT1* at very early developmental stages, when no S-lignin units have been yet deposited in *Arabidopsis*. Since *At*OMT1 has an affinity for quercetin *in vitro*, the role of this OMT in flavonoid metabolism and its induction after wounding and pathogen infection needs further investigation. The consequences of the absence of OMT1 from the lignin structural traits to the cell wall digestibility are similar to those observed in other plants. The screening of *Arabidopsis* mutants constitutes therefore a promising strategy to isolate new genes involved in lignin biosynthesis and secondary cell wall formation.

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